

Multistep Virus-Induced Leukemogenesis In Vitro: Description of a Model Specifying Three Steps Within the Myeloblastic Malignant Process

JEAN MICHEL HEARD,* SERGE FICHELSON, BRIGITTE SOLA, MARIE ANTOINETTE MARTIAL, BRUNO VARET, AND JEAN PAUL LEVY

Laboratoire Immunologie et Virologie des Tumeurs, Institut National de la Santé et de la Recherche Médicale U152, Hôpital Cochin, Paris, France

Received 11 July 1983/Accepted 7 October 1983

A helper-independent Friend leukemia virus was used to infect bone marrow cultures. This virus induces myeloblastic leukemia in mice after a long latency period. Infection of the bone marrow cultures resulted in the in vitro production of myeloblastic leukemogenesis after a long latency period. Three steps were observed in the evolution of the infected cultures, and permanent cell lines were derived at each step. This allowed us to individualize three successive events in the course of the myeloblastic transformation: (i) an abnormal responsiveness to the physiological hormone granulo-macrophagic colony-stimulating factor, (ii) the acquisition of growth autonomy, and (iii) the acquisition of in vivo tumorigenicity.

Naturally occurring as well as retrovirus-induced leukemias are long-latency diseases in mice. The same probably holds true for human leukemias. Therefore, experimental infections with viruses like Gross, Moloney, or the helper-independent Friend murine leukemia virus (F-MuLV) might provide animal models for the human disease. However, in virus-infected animals the molecular events leading from a normal hemopoietic progenitor to a leukemic cell are difficult to study. One working hypothesis is that cellular *onc* genes might be activated during the course of leukemogenesis. Possible mechanisms for this activation are the integration of a viral genome adjacent to cellular transforming sequences, as previously reported in chicken virus-induced leukemias (15), or chromosome translocations involving cellular *onc* genes which have been described in both murine plasmocytomas and human leukemias and B cell lymphomas (1, 4). In fact, it is likely that several cellular genes might be activated at different periods of the leukemogenic process (10, 20), and one can suppose that each *onc* gene activation results in the acquisition of unique cell properties characterizing each step of the process. Investigations of long-latency leukemias by using tumor cell lines derived during the overt leukemic period are not very informative about events occurring during the latent period. In particular, they are unable to elucidate serial activation of cellular genes leading to malignancy. We report here on an in vitro model of long-latency myeloblastic leukemia in which both the successive preleukemic steps and the overt leukemic period can be directly investigated on cultured cells. This in vitro long-latency leukemogenesis was produced by infecting cultured normal murine bone marrow cells with an F-MuLV stock which is known to induce a high incidence of myeloid leukemias in infected animals (16). Comparison of the time course and the cellular characteristics of both in vitro and in vivo-induced leukemias indicated that the in vitro leukemia was remarkably similar to the in vivo one. With this model, studies of the role of the different *onc* genes in myeloid leukemogenesis will be possible.

In vitro transformation of erythroid progenitors (6) or pre-B lymphocytes (28) have been unequivocally obtained by infecting cultures of bone marrow cells with the Friend virus

complex or the Abelson virus, respectively. In both cases, the rapid in vitro transformation mimicked the in vivo pathology of short latency. By contrast, an in vitro model of long-latency myeloid, erythroid, or lymphoid leukemias as induced by different murine retroviruses devoid of the *v-onc* gene has never been reported. Malignant myeloid cells seem to have been obtained, however, by Dexter et al. (8) in one unique bone marrow culture infected in vitro with the Friend virus complex, which is known to induce late myeloid leukemias in mice escaping the rapid erythroleukemia (3, 23). The precise characters of the malignant cells were difficult to establish since the tumor has not been maintained as a permanent cell line, neither in vivo nor in vitro. To better select the transformed cells, Greenberger et al. (12) established permanent cell lines from nonadherent cells of Friend virus complex-infected bone marrow cultures. This study was performed in the presence of WEHI-3-B conditioned medium, which provided several hemopoietic growth factors, including the granulo-macrophagic colony-stimulating factor (GM-CSF) (29). With this method, permanent cell lines were obtained, some of which induced myeloid leukemias in recipient mice. One can assume, therefore, that an in vitro transformation had occurred in these cultures, even though a definite karyotypic demonstration of the donor origin of the leukemias was not given. However, three characteristics suggest that the induced pathology might be different from long-latency myeloid leukemias induced in vivo by the Friend virus complex. (i) Cell lines were derived from the infected cultures as early as 8 to 10 weeks postinfection (12). By contrast, the latency of myeloid leukemias induced in vivo by the same virus exceeded 6 months (3, 23). (ii) In vitro-transformed cells always remained growth-factor dependent (12, 13), whereas leukemic cells derived from in vivo myeloid tumors grew as autonomous cell lines in in vitro culture whatever the origin of the leukemia (9, 16, 18, 19, 29). (iii) The spleen focus-forming virus component of the Friend virus complex was claimed to be responsible for the generation of these cell lines (11). By contrast, the helper-independent virus, rather than the spleen focus-forming virus component, was probably involved in myeloid leukemias induced in vivo by the Friend virus complex, since the helper virus alone induces such diseases (16, 21, 25).

* Corresponding author.

Bone marrow cultures were established as described by Dexter et al. (5). The contents of single femurs from (C57BL/6 × BALB/c)F1 female mice were flushed into 25-cm² flasks (Corning Glass Works, Corning, N.Y.) in 10 ml of alpha medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 15% fetal calf serum (FCS) and 10⁻⁷ M hydrocortisone (Sigma Chemical Co., St. Louis, Mo.). Cultures were incubated at 37°C in a 5% CO₂ atmosphere. Virus infection was performed 3 weeks later, when a monolayer of adherent fibroblastoid, adipocytic, and macrophagic cells was constituted. Samples (10⁵ focus-inducing units) of the biologically cloned, helper-independent F-MuLV strain I⁻⁵ stock were incubated for 1 h on monolayer cells in 1 ml of medium. Fresh bone marrow cells were then added as previously to the cultures in 10 ml of fresh medium. Cultures were further refed twice a week by exchanging one-half of the medium. Cells collected with supernatants were pelleted, counted, and used for either morphological examination or establishment of secondary cultures. In the latter case, 0.5 × 10⁶ to 2 × 10⁶ cells removed from infected or control cultures were plated in 10 ml of alpha medium with 10% FCS in the absence of hydrocortisone. Refeeding of step 1 secondary cultures was performed by exchanging one-half of the culture medium twice a week. Step 2 cultures were obtained by serial passages of step 1 nonadherent cells. When established, step 2 cultures were passed three times a week in alpha medium with 5% FCS. Identification of cells was performed by May-Grünwald Giemsa and myeloperoxidase staining of cytocentrifugation smears. Cells removed from primary or secondary cultures were injected into preirradiated (450 rads) syngeneic animals. Each recipient was inoculated with 5 × 10⁶ to 2 × 10⁷ cells both intraperitoneally and subcutaneously. Mice were regularly examined for the presence of any tumor until 6 months postinoculation. Tumors removed from moribund animals were used for cytological studies, karyotypic analysis, and transplantation into secondary recipients. For karyotypes, cells were incubated 1 h at 37°C with colchicine (0.25 µg/ml; Houdé Laboratoire, Paris), lysed with a 75 mM KCl solution, and fixed in methanol-acetic acid (3/1). Banding techniques used were R banding and G banding. All mitoses analyzed were photographed, and a minimum of four karyotypes were established. H2 antigen typing was performed by complement-dependent cytotoxicity assay with anti-H2^b and anti-H2^d antisera prepared in our laboratory.

We infected long-term bone marrow cultures (LTBMC) (5) derived from (C57BL/6 × BALB/c)F1 mice with the biologically cloned, helper-independent F-MuLV strain I⁻⁵. This virus was initially isolated from the polycythemia-inducing Friend virus complex. It is devoid of any spleen cell focus-

forming virus or mink cell focus-forming virus component and remarkable by its myeloid tropism in vivo (16). In an attempt to select transformed cells, nonadherent cells from infected LTBMC have been serially subcultured in the absence of any exogenous growth factor. This resulted, in repeated experiments, in the establishment of permanent cell lines. Four steps can be distinguished in the evolution of I⁻⁵-infected LTBMC (Table 1).

Each step was characterized by the acquisition of one of the properties which specify the myeloid leukemic cells: abnormal responsiveness to the physiological differentiation-inducing protein GM-CSF (24), acquisition of growth autonomy, and acquisition of in vivo tumorigenicity. Cloned and uncloned cell lines have been derived at each step and stored in liquid nitrogen for further investigations.

Step 0. Step 0 is defined by the absence of difference between infected and uninfected primary cultures. However, whereas all uninfected primary cultures (20 of 20) died before week 21, 13 of 20 (65%) infected primary cultures could be maintained more than 1 year.

Step 1. Step 1 is defined by the capacity of primary culture nonadherent cells to initiate secondary cultures. These secondary cultures were established in the absence of any exogenous growth factor but depend on the presence of an adherent cell layer. When transferred to a new culture flask, nonadherent cells first developed a fibroblastoid monolayer, followed several days later by the appearance of granulocytic cells in the supernatant. Between weeks 15 and 25, establishment of adherent layer-dependent secondary cultures became possible in 13 of 20 infected cultures. The same phenomenon was never observed in controls. The proportion of immature myeloblastic cells in secondary cultures progressively increased during step 1, whereas in contrast, the macrophages were increasingly more prevalent in uninfected primary cultures.

When plated in semisolid agar cultures in the absence of colony-stimulating factor (CSF), step 1 cells formed only few clusters. When various sources of CSF, including purified lung GM-CSF (2), were added to the cultures, colony formation increased dramatically (data not shown). Responsiveness to postendotoxin CSF by step 1 cells differed from that of normal bone marrow cells or step 0 cells (Fig. 1): (i) the number of cloning cells was higher at step 1; and (ii) whereas step 0 colonies were primarily mature granulocytic and macrophage cells, both mature granulo-macrophagic and immature myeloblastic cells were present within each step 1 colony. The immature cells increased from week to week. Furthermore, step 1 cell colonies could be replated and recloned in the presence of purified lung GM-CSF, and they generated continuous cloned cell lines in liquid suspen-

TABLE 1. Time course of I⁻⁵ (F-MuLV)-infected cultures and controls

Step (characteristics)	I ⁻⁵ -infected cultures			Uninfected controls	
	No. of cultures	Delay after infection (wk)	Mean duration of the step (wk)	No. of cultures	Mean duration of the step (wk)
0 (Primary LTBMC)	20		18 ± 5.7	20	16 ± 5.2
1 (Adherent layer-dependent secondary cultures)	13	18 ± 5.7	18.1 ± 6.2	0	
2 (Nontumorigenic autonomous cell lines)	8	36 ± 8.2	22.6 ± 7	0	
3 (Tumorigenic autonomous cell lines)	4 ^a	58.7 ± 10 ^a	Permanent	0	

^a Data are from the four cell lines which have been hitherto tested for tumorigenicity.

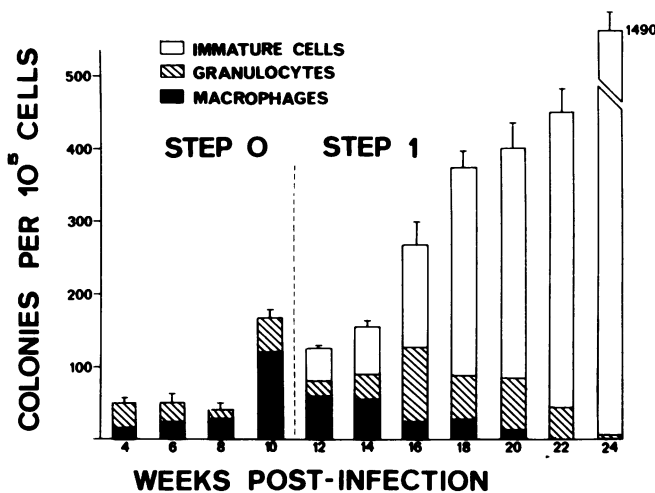


FIG. 1. Colony formation by step 0 and step 1 nonadherent cells in the presence of CSF. Nonadherent cells removed from either primary cultures (step 0) or adherent layer-dependent secondary cultures (step 1) were counted and plated at a final cell concentration of 7×10^5 cells per ml in 0.3% agar cultures with 10% FCS in alpha medium in 32-mm petri dishes (Corning). Cultures were supplemented with 3.5% serum from postendotoxin mice as a source of CSF (22) and incubated in a 5% CO_2 atmosphere at 37°C . Colonies were scored at day 6 under an inverted microscope. These culture conditions allowed the induction of 150 to 200 granulo-macrophagic colonies per 10^5 plated normal bone marrow cells. Identification of cells present within single colonies was performed by micropipetting, cytocentrifugation, and May-Grünwald Giemsa staining. More than 15 colonies were randomly picked up from each culture dish. Single colonies were sterilely picked up and suspended in culture medium for replating and establishment of suspension cell lines. Results are the mean \pm standard deviation of duplicate experiments.

sion cultures supplemented with a source of GM-CSF. Such supplementation was not necessary for the growth of uncloned step 1 nonadherent cells, probably because the adherent cell layer present in uncloned cultures produced the CSF-related factor(s) (14; data not shown). These results indicated that step 1 cells exhibited an abnormal responsiveness to GM-CSF which promoted their proliferation instead of their differentiation.

Step 2. Step 2 is defined by the acquisition of growth autonomy in the absence of adherent cells. At 26 to 49 weeks postinfection, cells were able to grow as pure suspension cell lines in 8 of the 13 step 1 cultures. At step 2, the lines were constituted almost exclusively of blast cells and could be easily subcloned. The use of serial subcultures in the absence of exogenous growth factor favored the selection of autonomous cells which appeared, under these conditions, after a long latency (36 ± 8.2 weeks) but with high frequency (8 of 20; 40%). This method eliminated any confusion with the establishment of factor-dependent, nonmalignant cell lines with mastocytic or promyelocytic characteristics, which are frequently obtained from uninfected LTBM (7, 26).

Experiments have been done to further study the acquisition of growth autonomy. Cells derived from *in vivo*-induced myeloid leukemias have been shown to stimulate their own proliferation (16) through the autocrine production of a CSF-like factor (manuscript in preparation). This property resulted in a cloning efficiency that increased with the number of plated cells. Similar experiments were performed with cells from *in vitro*-infected cultures (Fig. 2). Nonadherent cells were plated in semisolid agar cultures at the different steps of

their evolution, in the absence of exogenous stimulating factor. Step 1 cells never cloned in these culture conditions. Early step 2 nonadherent cells were able to clone when plated at relatively high cell concentrations, whereas late step 2 cells and step 3 cells (which were able to graft *in vivo*; see below) evidenced a much higher cloning efficiency, which increased with the number of plated cells. These results confirmed the probable capacity of autostimulation beginning in step 2 cells. It is remarkable that this property appeared at the same time as the ability to grow in suspension cultures in the absence of fibroblastoid cells, which are known to secrete growth factors for normal myeloid progenitor cells (CSF) (14).

Step 3. Nonadherent cells from infected LTBM were regularly inoculated into preirradiated syngeneic recipient mice since step 1. Step 1 and step 2 cells were never tumorigenic. Acquisition of *in vivo* engraftment defines step 3. It could be demonstrated in all four cell lines which have been hitherto tested. Delays between the passage to complete autonomy *in vitro* (step 2) and the appearance of an oncogenic potency (step 3) varied from 15 to 25 weeks depending on the cell line, which means a period of 48 to 65 weeks between *in vitro* virus infection and malignant transformation. In (C57BL/6 \times BALB/c)F1 mice infected as young adults with I⁻⁵ virus, the incidence of myeloblastic leukemias was 50%, and the delay before the induction of leukemias varied between 40 and 48 weeks. This means total delays of 50 to 60 weeks before permanent leukemic cell

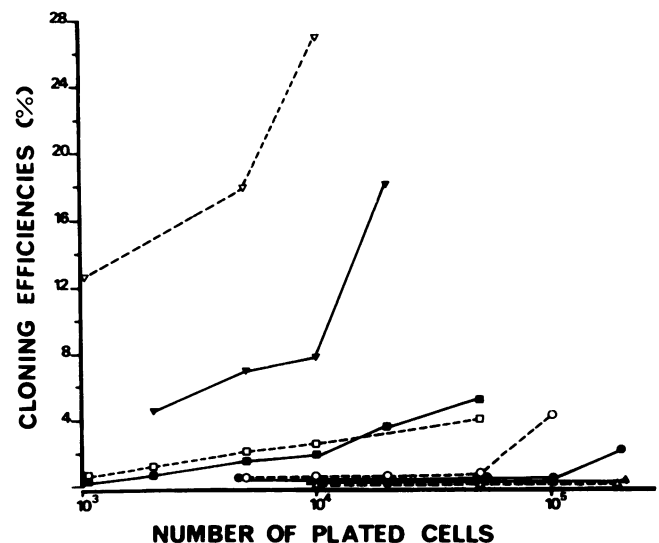


FIG. 2. Acquisition of growth autonomy by step 2 cells. Cloning efficiencies in the absence of exogenous stimulating factor are expressed in terms of the number of plated cells. Step 1, step 2, and step 3 cells from two independent *in vitro*-infected cultures were frozen and stored in liquid nitrogen. Cells were simultaneously thawed to investigate their cloning ability in the same assay. Cells were cultured for 2 weeks after thawing; then nonadherent cells were removed, pelleted, counted by trypan blue exclusion, and plated in 32-mm petri dishes (Corning) in 0.3% agar cultures with 10% FCS in alpha medium. Cultures were incubated in a 5% CO_2 atmosphere at 37°C , and colonies were scored at day 6 under an inverted microscope. Data are the mean of duplicate determinations. Symbols: Δ , \blacktriangle , step 1 cells; \circ , \bullet , early step 2 cells; \square , \blacksquare , late step 2 cells; ∇ , \blacktriangledown , step 3 cells. Some colonies were removed by micropipetting for identification after cytocentrifugation and staining with May-Grünwald Giemsa and myeloperoxidase. All examined colonies were constituted of 100% immature myeloperoxidase-positive cells.

lines could be established from these leukemias. Tumors were obvious within 5 to 8 weeks following the *in vivo* inoculation of large numbers of cultured cells. Subcutaneous tumors were only obtained with two cell lines. The other two gave both local tumors and generalized leukemias very similar to the myeloid diseases induced *in vivo* by the I^{-5} virus (16). In all cases, the cell morphology and myeloperoxidase activity were typical of myeloblasts, and these cells were transplantable in preirradiated recipient animals. Serial transplantations could be achieved by injecting low numbers of cells. Delays before obvious tumor induction were then comparable with those needed for *in vivo* engraftment of myeloblastic cell lines derived from leukemias induced *in vivo* by I^{-5} virus (25 to 40 days).

The definite proof that the malignant cells were derived from infected LTBM was obtained by the following two experiments. (i) Complement-dependent cytotoxicity. All autonomous cell lines were shown to be $H2^{bd}$ positive, as were the original *in vitro*-infected cultures. (ii) Karyotypic analysis of female tumor cells developed in male recipients revealed the absence of a Y chromosome and showed different chromosome distributions according to the cell line (data not shown). This proved that in each case the leukemia was derived from engrafted cells and was not induced *in vivo* by retroviruses. Furthermore, this ruled out the possibility of contamination of cultures by already established (*in vivo*-induced) cell lines, since no tumor of the $H2^{bd}$ phenotype was being maintained in our laboratory. In addition, it demonstrated that the four malignant cell lines were all different from each other.

Involvement of I^{-5} virus in the establishment of autonomous and tumorigenic myeloblastic cell lines appeared very likely since similar results were never found in uninfected cultures (20 of 20). Moreover, previous experiments in which the Moloney virus was used to infect LTBM always remained negative (17). Preliminary results with another helper-independent Friend leukemia virus (clone 201) (27), which induces lower incidences of myelogenous leukemias *in vivo* than I^{-5} (P. Tambourin, unpublished data), suggested that it induced rare myeloblastic transformation *in vitro*. The fact that the malignant cells and engrafted leukemias observed in our experiments were identical to their *in vivo* counterpart induced by I^{-5} is further support for the involvement of this agent in the induction of leukemia. The absence of any spleen focus-forming virus in all experiments clearly distinguished this system from previously published descriptions of *in vitro* myeloid transformation (8, 11, 12). This fact could perhaps explain some discrepancies between these systems, especially the complete growth autonomy of transformed cells and the much longer latency observed in our experiments.

The *in vitro* transformation system we report here provides an example of a long-latency leukemogenic process occurring entirely in culture. It allows identification of several steps within the myeloid malignant transformation, each of them characterized by the acquisition of one of the leukemic cell properties. Two properties are acquired successively during the preleukemic period: the abnormal responsiveness of step 1 cells to their physiological differentiation-inducing protein GM-CSF, which promotes cell proliferation instead of differentiation, and the autonomous growth pattern of step 2 cells. Experiments are in progress to determine whether this autonomy is due to the autocrine production of a CSF-related factor, as shown for the *in vivo*-transformed myeloid leukemic cells (16). The acquisition of *in vivo* engraftment by the *in vitro*-infected cells defines the

overt leukemic period at step 3. Cloned and uncloned cell lines have been isolated at each step and provide unique material for further investigations of eventual *onc* gene activations and specific integration sites of F-MuLV proviruses.

ACKNOWLEDGMENTS

We thank S. Gisselbrecht and P. Tambourin for helpful discussions and H. Gralnick for reviewing the manuscript. We are also grateful to A. W. Burgess for providing purified lung GM-CSF.

This work was supported by grants from Délégation Générale de la Recherche Scientifique et Technique (81-L-0729) and Université René Descartes (928).

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